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CONTRACT NO: DAMD17-91-Z-1027

TITLE: GLYCOPHINGOLIPIDS AS PUTATIVE RECEPTORS OF
STAPHYLOCOCCAL ENTEROTOXIN B IN HUMAN KIDNEY
PROXIMAL TUBULAR CELLS

PRINCIPAL INVESTIGATOR: SUBROTO CHATTERJEE

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13. ABSTRACT (Maximum 200 words) We have investigated the binding of ¹²⁵ I staphylococcal enterotoxin-B (SEB) to glycosphingolipids (GSL) from cultured human kidney proximal tubular cells employing GSL overlay technique and microplate quantitative assay. Structural studies of the putative GSL receptor for SEB was performed employing high performance liquid chromatography (HPLC), gas liquid chromatography, mass spectrometry (GC-MS) of Trimethylsilyl derivatives of methylglycosides, fatty acids and sphingosines. Permethylatation analysis of sugars was carried out employing GC-MS. Our results are: 1. Out of twelve GSL present in human kidney, only a diglycosyl ceramide specifically bound to SEB. 2. The chemical composition of the putative receptor: carbohydrates, glucose, galatose, sphingosines, d18:2, d23:0, fatty acids, C16:0, C18:1				
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FOREWORD

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Grant No. DAMD17-91-Z-1027

MID TERM PROGRESS REPORT

From June 1, 1991 - November 30, 1992, research activities have been aimed at the following areas.

Establishing human kidney proximal cell (PT Cell) culture in the laboratory from human cadaver kidneys.

Essentially the same procedure (1) as described in the research project was followed to culture human PT cells. Confluent cultures of PT cells were subcultured employing digestion with trypsin and passaged up to 2-3 times. Confluent cultures of PT cells were harvested and stored frozen for the isolation of GSL.

Isolation of Glycosphingolipids (GSL) from human kidney/PT cells and other tissues.

GSL were isolated from human kidney or PT cells by the procedure described previously. (2) GSLs were also prepared from rat kidneys, human brain, erythrocytes and human intestine as above. The GSLs were separated by thin layer chromatography on silica gel-G HPTLC plates (Merck) using chloroform-methanol-water (55:25:4 v/v) as the solvent system, and were identified using aniline diphenylamine (DPA) reagent. Figure 1 shows the separation of human kidney GSL on a high performance thin layer chromatography (HPTLC) plate. The major GSL species observed in human kidney were glucosylceramide (GlcCer), lactosylceramide (LacCer), trihexosylceramide (Gb₃Cer) and tetrahexosylceramide (Gb₄Cer) (Figure 1).

SEB Receptor Identification

Staphylococcal enterotoxin-B (SEB) was radiolabelled with ¹²⁵I using Iodogen and was used for overlaying procedures (3). GSL from human kidney or PT cells were separated as described above and identified using DPA reagent (Fig. 2A). A duplicate plate was incubated with ¹²⁵I-SEB for binding to the individual glycosphingolipids. Autoradiograms of the plate were then analyzed for binding activity. Figure 2B, an autoradiogram, shows that ¹²⁵I-SEB bound to human kidney GSL, corresponding in chromatographic migration to LacCer. ¹²⁵I-SEB binding to GSLs derived from rat kidney, human brain or human intestine was not observed.

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Binding of SEB to purified kidney GSL (SEB Receptor).

The GSL fraction showing binding with ^{125}I -SEB on HPTLC plate was purified as described and referred to as SEB receptor (SR). The binding of SEB to this receptor was assayed by measuring the attachment of ^{125}I -SEB to this putative receptor (SR) immobilized on microtiter plates (Immunolon wells) (4). The ^{125}I -SEB binding to GSL derived from human kidney (HKGSL), the SR, LacCer and the GSL derived from rat kidney (RKGSL) is shown in Figure 3. HK GSL and the SR, both, showed significant binding to ^{125}I -SEB. Maximal binding of SEB to HKGSL and SR occurred at a concentration of 100 ng/well and 20 ng/well, respectively. At this concentration, the SR binding to SEB was 5-fold more than HK GSL. A significant decrease in ^{125}I -SEB binding to HKGSL was observed at a concentration of 100 ng/well and above. A similar inhibition was observed with SR at the concentration of more than 20 ng/well. This observed decrease in binding could be due to the formation of multilamellar layers in the well at these GSL concentrations which have been reported to result in the inhibition of the receptor binding (4). Synthetic LacCer also bound some ^{125}I -SEB; at a GSL concentration of 20 ng/well it bound ^{125}I -SEB in the order of 26 fold less than purified SEB receptor GSL, (Figure 3). Increasing the concentration of synthetic LacCer in the assay mixture did not increase its ^{125}I -SEB binding further, showing saturable binding of this GSL at low concentrations. RKGSL did not bind to ^{125}I -SEB at these concentrations (Figure 3). SEB did not bind to LacCer prepared from rat kidneys (data not shown).

Specificity of ^{125}I -SEB Binding

The specificity of SEB binding to SR was assessed further employing various structurally defined glycosphingolipids in Table 1. These were GalCer, GlcCer, Gbose₄Cer, GM₁, GT_{1b}, and sulfatide (SO₄-GalCer). At GSL concentrations on the order of 10-1000 ng/well, a marginal binding of ^{125}I -SEB to these GSLs were seen. At low concentrations of GSL, ^{125}I -SEB did not bind to any of these GSLs. (Data not shown).

Characterization of SEB Receptor

The putative GSL receptor for SEB (SR) is at present being characterized further.

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High Performance Liquid Chromatography (HPLC) of SEB Receptor

Purified SEB receptor GSL was perbenzoylated by the method of Ullman and McCluer (5) and a suitable aliquot was subjected to HPLC on a Spherisorb Si-5 column with detection at 230 nm as described (6). In figure 4 (B) a HPLC chromatogram of the purified SR is presented. It resolved into two main peaks, with retention times on the order of 8.49 min. and 8.74 min, respectively. The standard LacCer (stearoyl LacCer) under similar conditions, also resolved into two peaks, having lower retention times on the order of 8.14 and 8.39 Figure 4 (A). The SR GSL was quantified using a standard curve prepared with authentic LacCer.

Gas Chromatography-Mass Spectrometry (GC-MS) of SEB Receptor

The SR was subjected to acid catalysed methanolysis. The methylglycosides, methyl fatty acids and methyl sphingosines were derivatized employing trimethylchlorosilane and analyzed on an Ion Trap Detector-800 (ITD-800) GC-MS using DB-5 capillary column (0.25 X 30 m). Table 2 shows the percent fatty acid, sphingosine composition and sugar ratio of SR. The GC-MS chromatogram of SR is shown in Figure 5. The preliminary data shows it to contain mainly three fatty acids, namely methyl palmitate (C16; molecular weight 270.46); methyl elaidate (C 18:1; molecular weight 296.48) and methyl stearate (C18; molecular weight 298.51); two sugars, namely glucose and galactose and three sphingosine bases (d18:2, d22:2, d23:0). Further GC-MS analysis of this GSL is in progress.

Publications

The following papers and abstracts were published during June 1, 1991 - November 30, 1992.

Publications:

1. Chatterjee, S.; Jett, M. "Glycosphingolipids: The putative receptor for staphylococcus-aureus enterotoxin-B in human kidney proximal tubular cells." Mol.Cell. BioChem. 113 :pp.25-31 (1992)
2. A manuscript relevant to the work presented here is under preparation.
3. A review article entitled "Glycosphingolipids as putative receptors." is under preparation.

Grant No. DAMD17-91-Z-1027

Abstracts:

1. Chatterjee, S.; Jett, M. "Glycosphingolipids as putative receptors for Staphylococcus-aureus toxin-B in cultured human proximal tubular cells." FASEB. J. 5 :pp.2, 629. (1991).

References:

1. Chatterjee, S.; Trifillis, AL.; Regec, AL.; (1987) Can. J. Biochem. Cell. Biol. 65: pp.1,049

2. Esselman, WA.; Laine, RA. and Sweeley, CC. (1972) Methods Enzymol. 28: pp.140

3. Karlsson, KA. and Stromberg, N. (1987) Methods Enzymol. 138: pp.220

4. Karlsson, KA. and Stromberg, N. (1987) Methods Enzymol. 138: pp.227

5. Ullman, MD. and McCluer, RH. (1987) Methods Enzymol. 138: pp.117

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Table 1. Structure of Glycosphingolipids Used to Determine Specificity of ¹²⁵I-SEB Binding

- | | | |
|----|---|------------------------------|
| 1. | Lactosyl Ceramide | Gal(B1-4)Glc-Cer |
| 2. | Glucosyl Ceramide | Glc-Cer |
| 3. | Galactosyl Ceramide | Gal-Cer |
| 4. | Globoside | |
| | GalNAc (B1-4)Gal(B1-4)Gal(B1-4)GlcB1-1Cer | |
| 5. | Trisialoganglioside: | |
| | NeuAc2-3Gal(B1-3)GalNAc(B1-4)Gal(B1-4)Glc(1-1)Cer | |
| | | 3

-NeuAc2-8 - NeuAc2 |
| 6. | Monosialoganglioside: | |
| | NeuAc2-3Gal(B1-4)Glc(B1-1)Cer | |
| 7. | Galactosyl Sulfatide | SO ₄ -Gal-Cer |

Table 2. GC-MS Data of Purified Receptor GSL

1.	Carbohydrates:	Galactose	10.28 nmol	
		Glucose	14.12 nmol	
		Gal/Glc	1:1:37	
2.	Sphingosine:			
		d18:2	d22:2	d23:0
		% 45.34	8.04	46.62
3.	Fatty Acid			
		C16	C18:1(8-ene)	D18
		% 29.96	30.78	39.24

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Legends to Figures

Figure #1 - HPTLC of human kidney glycosphingolipids (GSLs) on an HPTLC (silica gel G) plate using chloroform-methanol-water (65:24:4 v/v). The plate was dried in air and developed with aniline diphenylamine reagent. Lane 1 - human kidney GSL; Lane 2 - cultured proximal tubular cell GSL.

Figure #2 - Binding of ^{125}I -labelled Staphylococcal enterotoxin-B (SEB) to glycosphingolipids (GSLs) separated by high performance thin layer chromatography (HPTLC). A - GSLs detected with aniline diphenylamine reagent; B - GSL detected by radiography (exposure time: 18 hrs.) employing ^{125}I -SEB (1×10^5 cpm/ml; incubation for 4 h at room temperature). Lanes 1 and 6 - Human kidney GSL; Lanes 2 and 5 - PT cell GSL; Lanes 3 and 4 - rat kidney GSL.

Figure #3 - Binding of ^{125}I -labelled staphylococcal enterotoxin-B (SEB) to glycosphingolipids (GSL) coated on microtiter wells. Data are expressed as mean values of triplicate determinations. The X-axis indicates the amount of GSL coated to microtiter wells.

Figure #4 - HPLC analysis of perbenzoylated (GSL) A - LacCer; B - Staphylococcal enterotoxin-B receptor (SR). GSLs were benzoylated, dried, suspended in hexane and injected.

Figure #5 - Mass chromatograms (5A - 5C) of staphylococcal enterotoxin-B receptor (SR). 5 (A) mass chromatogram of SR carbohydrates and sphingosine; 5 (B) mass spectrum of SR sphingosines; 5 (C) mass chromatogram of fatty acids.

Figure #6 - Mass spectrum (6A - 6C) of SR fatty acids. 6 (A) C16, methyl palmitate; 6 (B) C18:1, methyl elaidate; 6 (C) C18:0, methyl stearate

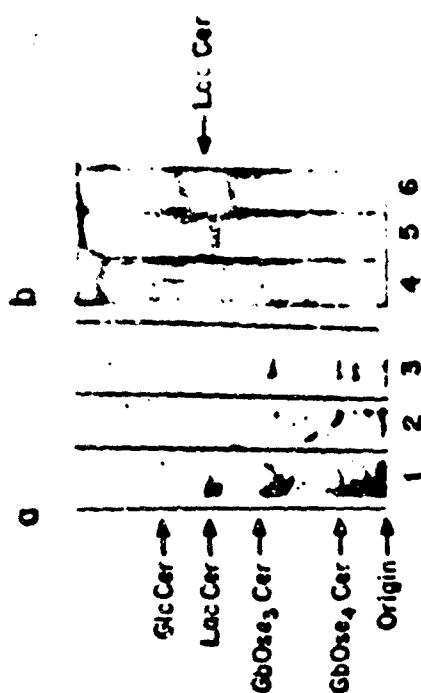


Figure 2

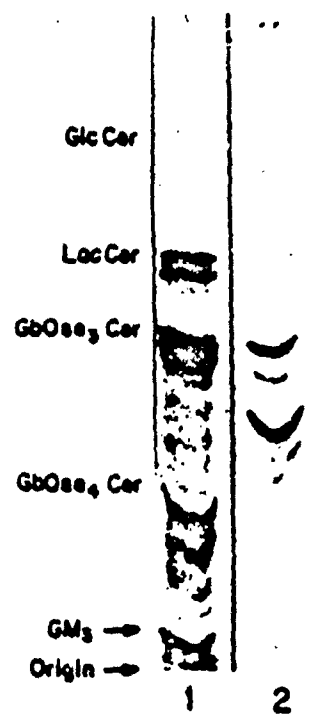


Figure 1

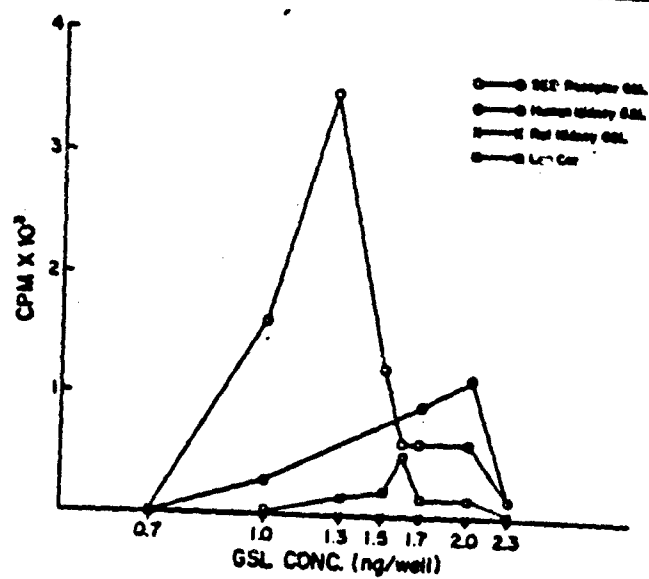


Figure 3

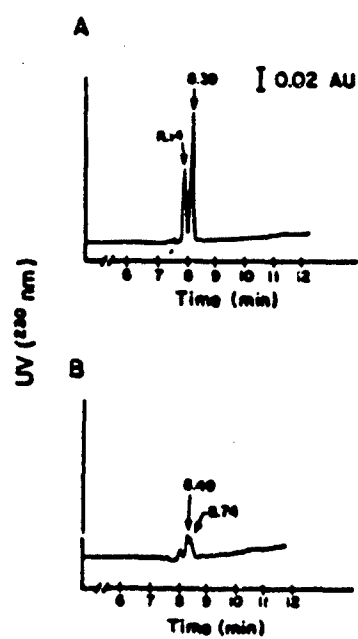


Figure 4

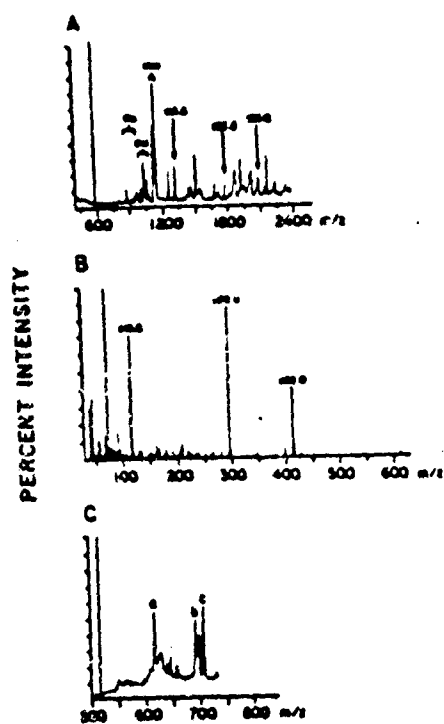


Figure 5

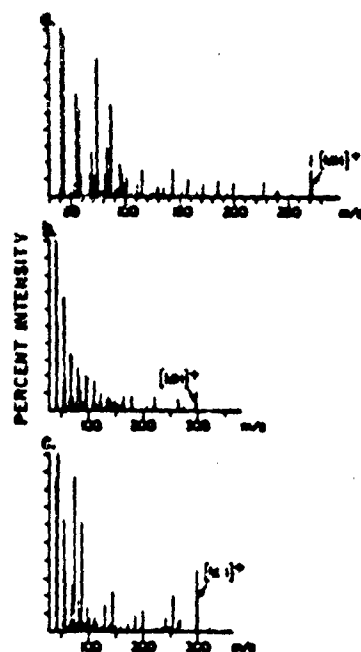


Figure 6

Glycosphingolipids: The putative receptor for staphylococcus aureus enterotoxin-B in human kidney proximal tubular cells

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Received 30 August 1991, accepted 13 February 1992

Abstract

We have investigated the binding of ¹²⁵I-staphylococcal enterotoxin-B (SEB) in cultured human proximal tubular cells. We found that the binding of ¹²⁵I-SEB to PT cells was time and concentration dependent and competitively inhibited by antibody against SEB. Preincubation of cells with trypsin and neuraminidase or with fetuin did not significantly impair the binding of ¹²⁵I-SEB to such cells. In contrast, treatment with endoglycoceramidase completely inhibited the binding of ¹²⁵I-SEB to cells. Neutral glycosphingolipids exerted a concentration-dependent inhibition of ¹²⁵I-SEB binding to such cells, maximum inhibition (96% compared to control) occurred upon incubation of PT cells with neutral glycosphingolipids. Taken together, our studies indicate that SEB specifically binds to a neutral glycosphingolipid in PT cells. In contrast, staphylococcal enterotoxin-A and toxic shock toxin (TST-1) are bound to a protein in such cells. (*Mol Cell Biochem* 113: 25–31, 1992)

Abbreviations: SEB – Staphylococcal Enterotoxin-B; SEA – Staphylococcal Enterotoxin-A; TST-1 – Toxic Shock syndrome Toxin; GSL – Glycosphingolipid; PT – Proximal Tubular; LPDS – Lipoprotein Deficient Serum; PBS – Phosphate Buffered Saline

Key words: glycosphingolipids, kidney proximal tubular cells, staphylococcal enterotoxin-A, B, toxic shock syndrome toxin

Introduction

Staphylococcal enterotoxin-B (SEB)¹ is an important pathogen which causes severe diarrhea and death in experimental animals and man [1]. Recently, HLA-DR in human TCB cell lines were found to have high affinity binding for SEB, SEA and TST-1 [2]. The binding of SEA to class II major histocompatibility complex

(MHC) molecules of murine fibroblasts transfected with HLA class III has been noted [2, 3]. However, localization and biochemical tracer studies reveal that the kidney plays a major role in the uptake of the toxin prior to its potent effect on other organs [4, 5]. In particular, 75% of radiolabeled toxin given to monkeys

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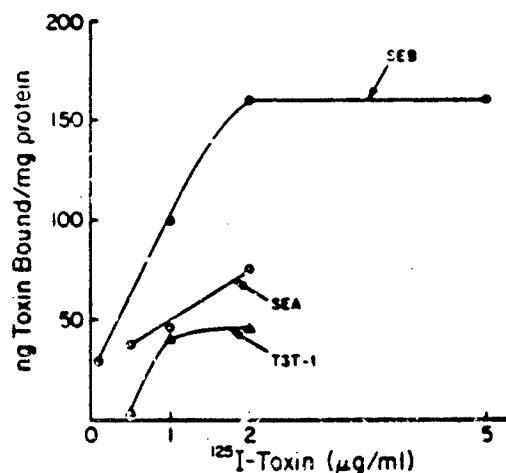


Fig. 1. Binding of ^{125}I -*Staphylococcus enterotoxin-B* (SEB) by cultured normal human proximal tubular (PT) cells. Human PT cells ($\times 10^5$) were seeded in 60×15 mm plastic petri dishes and grown in medium containing 10% fetal calf serum without antibiotics. On the sixth day of cell growth, cells were fed medium containing 1 mg protein/ml of lipoprotein deficient serum (LPDS) and incubated for 24 hours. Subsequently, fresh medium containing LPDS and $0.5 \mu\text{g/ml}$ of ^{125}I -SEB (specific activity 169 cpm/ng), ^{125}I -SEA (specific activity, 116 cpm/ng) and TST-I (specific activity, 104 cpm/ng) was added to one set of dishes. To another set of dishes, prior to the addition of ^{125}I -toxin, 20 fold excess of corresponding unlabeled toxin was added and incubation was continued for 2 hr at 37°C . Next, medium was removed and the cells were washed ten times with ice-cold phosphate buffered saline (PBS) for a period of about 30 min.

The samples were solubilized overnight with 1N NaOH and cell associated radioactivity and protein content was measured. All assays were pursued in duplicate dishes from two batches of PT cells and analyzed in duplicate. Specific binding i.e., binding in the absence of unlabeled toxin-binding in the presence of unlabeled toxin was calculated and plotted.

was found in PT cells in the kidney [1]. Because of the availability of well characterized human kidney PT cells in our laboratory [6], we have pursued studies to determine the biochemical nature of the receptor for SEB in such cells. Competitive high affinity binding studies of SEB with SEA and TST-I were pursued to reveal whether such toxins bound to similar or different binding domains in PT cells.

Materials and methods

Isotopes and chemicals

^{125}I (specific activity 644 MBq/ μg iodine) was purchased

from Dupont, New England Nuclear. All other biochemicals were purchased from Sigma Chemical Co., St. Louis. *Vibrio cholera* neuraminidase and *Rhodococcus* endoglycosceramidase were purchased from Calbiochem and Genzyme Corporation, Boston, respectively. "Phorcast" polyacrylamide gels and Rainbow protein markers (Mr 2,350–Mr 46,000) were purchased from Amersham Corporation. Human brain gangliosides and human kidney neutral glycosphingolipids were prepared in our laboratory [7] and characterized employing HPTLC and HPLC techniques [8]. Human low density lipoproteins (LDL; 1.019–1.063 gm/dl) and lipoprotein deficient plasma was obtained from the plasma of normal human volunteers by KBr density gradient ultracentrifugation [9]. Lipoprotein-deficient serum (LPDS) was prepared from lipoprotein-deficient plasma by precipitation with thrombin as described [10]. Such preparations were free from glycosphingolipids and cholesterol.

Preparation of SEB toxin and radio labeling with ^{125}I

Commercially available SEB, SEA and TST-I were labeled with ^{125}I using iodogen [11], solubilized in sample buffer and subjected to polyacrylamide gel electrophoresis on Phorcast gels at 12.5 milli amp/gel for 24 h at room temperature. Appropriate standard proteins of known Mr were also electrophoresed simultaneously. Following electrophoresis, a portion of the gel including the standard molecular weight proteins was sliced and stained with coomassie blue at 60°C for 5–10 min. The gel area corresponding to Mr 28,000 for SEB and SEA and 24,000 for TST-I was sliced, eluted and dialyzed. The material was freeze dried, solubilized and assessed for purity by SDS-PAGE analysis. Such preparations were free from contaminating proteins.

Cells

Cultured human PT cells were prepared from autopsy kidney as described previously [6]. Cells were trypsinized and seeded (1×10^5) in 60×15 mm plastic Petri dishes and grown for 6 days in medium containing 10% fetal calf serum and no antibiotics. On the 6th day, medium was removed, cells were washed with phosphate buffered saline (PBS) and incubation continued for 24 h in medium containing LPDS (1 mg protein/ml).

¹²⁵I-SEB binding assay

Unless otherwise described in the text, the following assay was adopted to measure the binding of ¹²⁵I-SEB to PT cells. Medium was removed from cells primed with LPDS. Next, fresh medium (2 ml) and ¹²⁵I-SEB (2 µg/ml) plus a twenty fold excess of unlabeled SEB was added and incubation continued for 2 h at 37°C. Next, the medium was discarded and the cells washed with 5 ml of PBS containing 0.2% bovine serum albumin (maintained at 4°C) and 5 times with PBS. The monolayer was solubilized in 1 N NaOH, protein and radioactivity was measured according to Lowry et al. [12] and scintillation spectrometry, respectively. Specific binding of ¹²⁵I toxin to PT cells was calculated by subtracting the data obtained in the absence of unlabelled toxin from the data obtained in the presence of 20 fold excess of unlabelled toxin.

Incubation of cells with enzymes

Cells preincubated with medium containing LPDS were further incubated for 5 min at 37°C with trypsin (0–500 µg/ml). The reaction was terminated by removing the enzyme solution from the dishes, washing the cells with PBS and incubation with soybean trypsin inhibitor followed by extensive washing with PBS. Trypsin treated cells were used in ¹²⁵I toxin and in ¹²⁵I-LDL binding studies [13]. Similarly, cells were incubated with neuraminidase (0.5 units/ml to 2.0 units/ml) and endoglycosidase (0.15 milliunits–0.6 milliunits/ml) for 1 h at 37°C, washed and the binding of ¹²⁵I-SEB pursued as described above.

Incubation of cells with glycosphingolipids

Cells preincubated with medium containing LPDS were further incubated with fresh medium containing glycosphingolipids and ¹²⁵I-toxin mixture. Glycosphingolipids were taken into a sterile glass tube and dried in N₂ atmosphere. Their medium containing LPDS was added, sonicated and suitable aliquots added to the assay mixture. After incubation for 2 hr at 37°C the assay was terminated and the binding of toxin to PT cells measured.

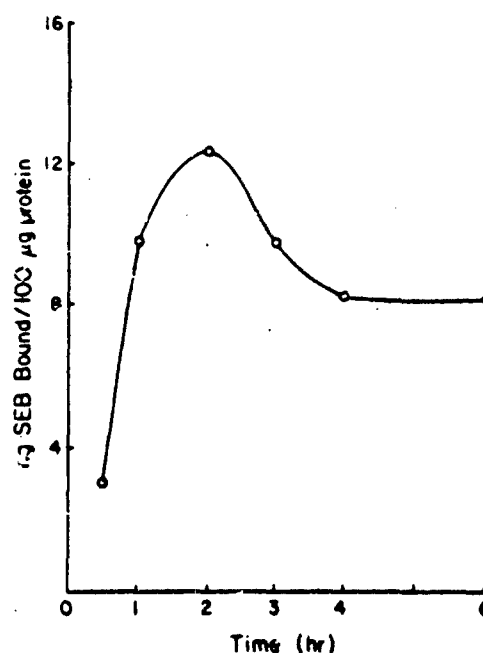


Fig. 2. Effect of time of incubation on the binding of ¹²⁵I-SEB to PT cells. The protocol of this experiment was identical to that described in Fig. 1 except that cells were incubated with 20 fold excess of the SEB and ¹²⁵I-SEB (2 µg/ml) for 1, 2, 4 and 6 hr at 37°C. The specific binding of ¹²⁵I-SEB to PT cells was calculated as described in Fig. 1. The data represents average values obtained from duplicate dishes from two separate batches of PT cells analyzed in duplicate.

Results

Binding of ¹²⁵I-staphylococcal enterotoxin-B (SEB) and other toxins by cultured normal human proximal tubular (PT) cells

The binding of ¹²⁵I-SEB to PT cells is shown in Fig. 1. Maximum high affinity binding occurred with 2 µg of SEB and TST-1 but not SEA per ml medium. The binding of ¹²⁵I-SEB to PT cell was saturated at higher concentration: 5 µg/ml medium. The ratio of binding of TST-1, SEA and SEB to PT cells was on the order of 1:1.5:3, respectively. The binding of ¹²⁵I-SEB to PT cells was time dependent (Fig. 2). For example, a linear increase in the binding of this toxin occurred up to 2 hr followed by a plateau after 4 hr. The inclusion of antibody against SEB in the assay mixture quantitatively inhibited the binding of this toxin to PT cells (Fig. 3). A

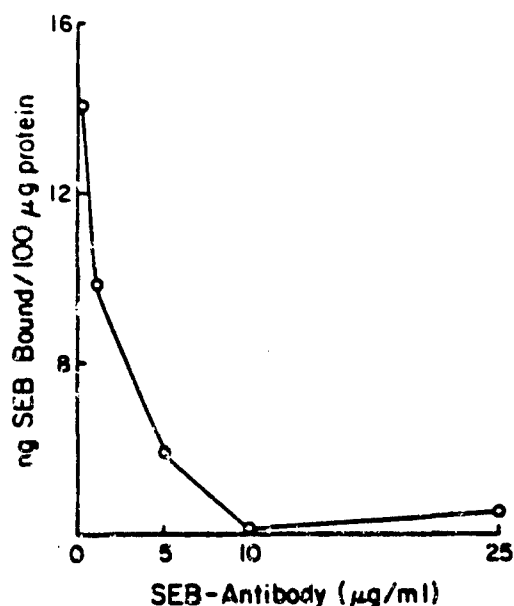


Fig. 3 Effect of antibody against SEB on the binding of ^{125}I -SEB in PT cells. To cultured PT cells 0, 1, 5, 10 and 25 μg of antibody against SEB was added prior to the addition of 2 $\mu\text{g}/\text{ml}$ of ^{125}I -SEB. Incubation was carried out for 4 hr at 37°C and the specific binding of ^{125}I -SEB to PT cells was measured. The data represents average values obtained from duplicate assays from two batches of PT cells analyzed in duplicate.

linear decrease in binding occurred upto 10 $\mu\text{g}/\text{ml}$ of SEB antibody.

Displaceable binding of toxins in PT cells

Displaceable binding assays employing a fixed amount of ^{125}I -toxin and increasing concentrations of unlabeled toxin revealed that, first, unlabeled SEB was unable to compete for the binding sites for ^{125}I -SEA and ^{125}I -TST (Fig. 4A). Similarly, unlabeled SEA was unable to compete with the binding of ^{125}I -SEB to PT cell receptors (Fig. 4B).

Effects of trypsin, neuraminidase and endoglycoceramidase on the binding of ^{125}I -SEB and other toxins in PT cells

Preincubation of cells with trypsin and neuraminidase maximally decreased ^{125}I -SEB binding in the order of

26.8% and 30.4%, respectively compared to control (Table 1). Under similar conditions, trypsin (500 $\mu\text{g}/\text{ml}$) inhibited 80% of ^{125}I -LDL binding to PT cells. Preincubation of PT cells with endoglycoceramidase did not impair the binding of ^{125}I -SEA or ^{125}I -TST-1 (data not shown). Preincubation with endoglycoceramidase (0.15–0.6 milliunit/ml) completely inhibited the binding of ^{125}I -SEB to PT cells (Table 1).

Effects of glycosphingolipids on the binding of ^{125}I -SEB to PT cells

Maximum inhibition of binding, (54.2% relative to control) occurred with 600 $\mu\text{g}/\text{ml}$ gangliosides (Table 2). In contrast, when 50 $\mu\text{g}/\text{ml}$ neutral glycosphingolipids were added in the incubation mixture, 96.7% inhibition of ^{125}I -SEB binding to PT cells occurred as compared to control.

Discussion

Our major findings in this report are: first, kidney proximal tubular cells have high affinity binding sites (receptors) for SEB; second, preincubation of cells with endoglycoceramidase and/or human kidney neutral glycosphingolipids markedly inhibited the binding of SEB to PT cells. Third, in contrast, endoglycoceramidase treatment did not impair the binding of ^{125}I -SEA or ^{125}I -TST-1 to PT cells. Moreover, these toxins were unable to competitively displace SEB from binding to PT cells.

Previous studies in experimental animals and man have suggested that the kidney, in general and proximal tubular cells in the kidney cortex in particular, may play a major role in the pathophysiology of SEB induced toxemia [4, 5].

We have found that PT cell can bind ^{125}I -SEB first, via a high affinity receptor mediated mechanism at low concentrations of toxin, as well as a nonsaturable receptor-independent mechanism at high concentration of SEB (Fig. 1). Competition experiments with unlabeled SEB, and antibody against SEB which quantitatively inhibited ^{125}I -SEB binding suggest that the receptors in such cells are specific for SEB. The inclusion of 3-iodotyrosine ($3 \times 10^{-4}\text{M}$), an inhibitor of deiodinase in the assay mixture, did not alter the binding of ^{125}I -SEB in PT cells (data not shown). These findings suggest that the binding of ^{125}I -SEB of PT cells is not due to the

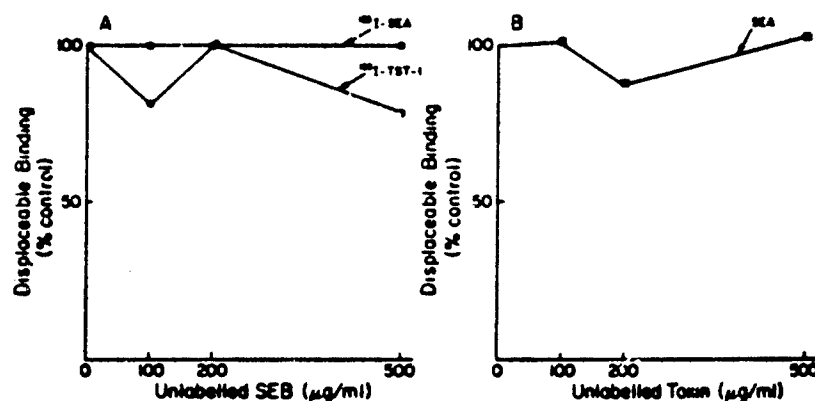


Fig. 4. Competitive binding of SEB, SEA and TST-I to PT cells. The protocol of this experiment was similar to the legend for Fig. 1 except that following the addition of ^{125}I toxin SEA and TST-I (Fig. A) unlabeled SEB (0–500 µg/ml) was added. Similarly to another set of dishes, ^{125}I -SEB plus (0–500) µg/ml unlabeled SEA was added and the displaceable binding of toxin was measured (Fig. B). The results represent data from one experiment analyzed in duplicate.

removal of ^{125}I by a deiodinase and the subsequent labeling of cells with ^{125}I . Rather, ^{125}I -SEB binding is due to the presence of receptors in PT cells.

To investigate the nature of the SEB receptor on PT cells, studies were pursued further. First, preincubation of cells with trypsin and neuraminidase followed by

Table 1. Effects of trypsin, neuraminidase and endoglycosidase on the binding of ^{125}I -SEB and ^{125}I -LDL in normal human kidney proximal tubular cells.

Enzyme	Binding (ng SEB bound/ 100 µg protein)	% Inhibition
^{125}I -SEB Control	11.2	0
Trypsin (50 µg/ml)	9.6	14.3
Trypsin (100 µg/ml)	8.9	20.6
Trypsin (500 µg/ml)	8.2	26.8
Neuraminidase* (0.5 units/ml)	7.5	33.1
Neuraminidase (1.0 units/ml)	8.5	24.2
Neuraminidase (2.0 units/ml)	7.8	30.4
Endoglycosidase** (0.15 milliunits)	0	100
Endoglycosidase (0.30 milliunits)	0	100
Endoglycosidase (0.60 milliunits)	0	100
^{125}I -LDL Control	10.0	0
Trypsin (500 µg/ml)	2.0	80

* One unit of neuraminidase releases 1 µmole of neuraminic acid/min.

** One unit of endoglycosidase releases 1 µmole of glucose from bovine brain gangliosides/min.

binding assays only moderately decreased ^{125}I -SEB binding. Under similar conditions, trypsin inhibited 80% of the binding of ^{125}I -LDL to PT cells compared to control. Previously, digestion of rat liver membranes with trypsin was found not to impair the binding of cholera toxin [14]. Second, SDS-PAGE analysis of PT cells incubated with ^{125}I -SEB for 2 hr revealed a labeled band at the dye front. In no instance did toxin bind to any other band visible in the coomassie brilliant blue stained gel. Third, inclusion of fetuin (250 µg/ml), a serum glycoprotein in the assay mixture, did not impair the binding of ^{125}I -SEB to PT cells.

Recently the binding of SEB to human T cells bearing

Table 2. Effects of human brain gangliosides and human kidney neutral glycosphingolipids on the binding of ^{125}I -SEB in normal human proximal tubular cells.

Glycosphingolipid	Binding (ng SEB bound/ 100 µg protein)	% Inhibition
Control	12.0	0
Gangliosides		
(30 µg/ml)	10.3	14.2
(150 µg/ml)	9.2	23.4
(300 µg/ml)	9.2	23.4
(600 µg/ml)	5.5	54.2
Neutral glycosphingolipids		
(5 µg/ml)	3.3	72.5
(10 µg/ml)	1.0	91.7
(50 µg/ml)	0.4	96.7

particular VB sequences as part of their receptors for major histocompatibility complex protein-associated antigen has been shown [3]. In another study a unique site on class II MHC proteins to which SEA, SEB and TST-1 binds was shown [2]. The reasons for the discrepancies in the above studies and ours is not clear presently. Since the amino acid sequence of TST-1 bears little resemblance with SEA and SEB, the binding of these three toxins to an identical site appears non-specific. Moreover, SEB is only 28% homologous to SEA and the binding of SEA to such sites is 10-13 times higher than SEB [2]. We also found that unlabeled SEB was unable to displace SEA or TST-1 from binding to PT cells. Similarly, SEA or TST-1 were unable to displace SEB from binding to PT cells. Moreover, in our studies, pretreatment with trypsin only partially decreased the binding of SEB to PT cells compared to preincubation of cells with glycosphingolipids or endoglycoceramidase which dramatically decreased 125 I-SEB binding to PT cells (see below). Our studies lead to the suggestion that there is a clear dichotomy in regard to the nature of receptor for SEA, TST-1 and SEB. In case of SEA and TST-1 the receptor is clearly a protein, in contrast, in case of SEB, the receptor is most probably a glycosphingolipid.

Several glycosphingolipids have been suggested to serve as receptors for various toxins. For example, GM₁ (ceramide-glucose-galactose-N-acetylneuraminic acid) and globotriosyl-ceramide (ceramide-glucose-galactose-galactose) serve as receptors for cholera toxin and verocytotoxin, respectively [14, 15]. Similarly, a large number of commonly occurring bacteria and pathogenic bacteria have been found to bind specifically to lactosylceramide (Ceramide-glucose-galactose) [19]. We investigated whether glycosphingolipids may also serve as a putative receptor for SEB in PT cells. First, we found that human kidney neutral glycosphingolipids are a potent inhibitor of SEB binding to PT cells. Such findings are in agreement to previous reports suggesting neutral glycosphingolipids as probable receptors for toxins. Furthermore, preincubation of cells with endoglycoceramidase resulted in complete inhibition of 125 I-SEB binding to PT cells. This enzyme specifically cleaves the glycosyl moiety from glycosphingolipids [22]. Finally, our preliminary studies reveals direct binding of SEB to a human kidney neutral glycosphingolipid. Taken together our findings suggest that most probably a neutral glycosphingolipid in PT cells serves as a putative receptor for SEB. Further studies are under way in our laboratory to determine the structure of the putative

glycosphingolipid receptor and to establish structure function relationships.

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